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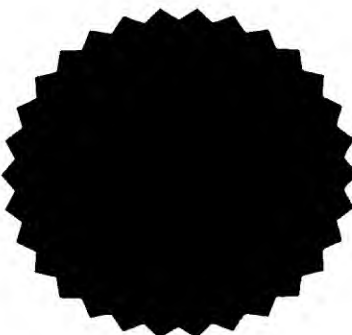
I hereby certify that annexed is a true copy of the Provisional Specification as filed on 27 January 1999 with an application for Letters Patent number 333928 made by COLIN R GREEN and DAVID L BECKER.

Dated 18 January 2000.

Neville Harris  
Commissioner of Patents

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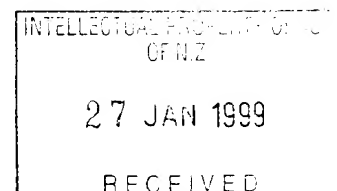
PATENTS ACT 1953

**PROVISIONAL SPECIFICATION**

**FORMULATIONS**

We, **DAVID L BECKER**, C/o UniServices House, 58 Symonds Street, Auckland 1001, New Zealand and **COLIN R GREEN**, C/o UniServices House, 58 Symonds Street, Auckland 1001, New Zealand do hereby declare this invention to be described in the following statement:

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(followed by page 1A)



**FORMULATIONS**

This invention relates to formulations for use in therapeutic and/or cosmetic treatments, particularly those in which a localised disruption in direct cell-cell  
5 communication is desirable.

**BACKGROUND**

Gap junctions are cell membrane structures which facilitate direct cell-cell  
10 communication. A gap junction channel is formed of two hemichannels (connexons), each composed of six connexin subunits. These connexins are a family of proteins, commonly named according to their molecular weight.

An ability to control connexin expression (and in particular to downregulate it)  
15 would therefore provide an opportunity to modulate cell-cell communication within a patient for therapeutic and/or remedial purposes. However, as a number of connexin proteins are expressed widely throughout the body, a general downregulatory effect is undesirable in inducing a therapeutic effect at a specific site.

20 Anti-sense deoxyoligonucleotides have considerable potential as agents for the manipulation of specific gene expression (reviewed: Stein *et al.*, 1992; Wagner 1994). However, there remain difficulties which need to be overcome. These include the short half life of such oligonucleotides (unmodified phosphodiester  
25 oligomers typically have an intracellular half life of only 20 minutes owing to intracellular nuclease degradation (Wagner 1994)) and their delivery consistently and reliably to target tissues.

It was with the intent of at least partially overcoming these difficulties that the  
30 applicants devised the present invention.

**SUMMARY OF THE INVENTION**

Accordingly, in a first aspect, the invention provides a formulation for use in  
35 therapeutic and/or cosmetic treatment, which formulation comprises:

at least one anti-sense deoxyoligonucleotide to a connexin protein; and

a sustained release vehicle for said deoxyoligonucleotides.

5 In one preferred form, the formulation contains deoxyoligonucleotides to one connexin protein only. Most preferably, this connexin protein is connexin 43.

Alternatively, the formulation contains deoxyoligonucleotides to more than one connexin protein. Preferably, one of the connexin proteins to which  
10 deoxyoligonucleotides are directed is connexin 43.

Conveniently, the deoxyoligonucleotide to connexin 43 is selected from:

15 GTA aTT gCC gCA GGA GGA ATT GTT GCT GTC; and  
GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT

Preferably, the sustained release vehicle is, or includes, a gel.

Conveniently, the gel is a pluronic gel, most preferably Pluronic F-127.

20 Conveniently, the formulation further includes a surfactant to assist with deoxyoligonucleotide cell penetration.

In a further aspect, the invention provides a method of site-specific downregulation  
25 of connexin protein expression for a therapeutic and/or cosmetic purpose which comprises administering a formulation as defined above to a site on or within a patient at which said downregulation is required.

In still a further aspect, the invention provides a method of reducing neuronal cell  
30 death which would otherwise result from a neuronal insult to a specific site in the brain of a patient which comprises the step of administering a formulation as defined above to said site to downregulate expression of connexin protein(s) at and immediately adjacent said site.

35 Preferably, the formulation is administered to reduce neuronal loss due to physical trauma to the brain.

Conveniently, the formulation is administered in a sufficient amount to downregulate expression of said connexin protein(s) for at least 24 hours post-administration.

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In yet a further aspect, the invention provides a method of promoting wound healing in a patient which comprises the step of administering a formulation as defined above to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

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Usually, the wound will be the result of trauma. It may however be the result of surgery.

15

In yet a further aspect, the invention provides a method of decreasing scar formation in a patient who has suffered a wound which comprises the step of administering a formulation as defined above to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

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Again, the wound may be the result of trauma or surgery, with the formulation being applied to the wound immediately prior to surgical repair and/or closure thereof.

#### DESCRIPTION OF THE INVENTION

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As broadly defined above, the focus of the invention is on site-specific downregulation of connexin expression. This will have the effect of reducing direct cell-cell communication at the site at which connexin expression is downregulated, which gives rise to numerous therapeutic/cosmetic applications as described below.

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The downregulation of connexin expression is based generally upon the anti-sense approach, and more particularly upon the use of anti-sense deoxyoligonucleotides (ODN). These ODN target the connexin protein(s) to be downregulated.

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The connexin protein or proteins targeted by the ODN will be dependent upon the site at which downregulation is to be effected. This reflects the non-uniform make-

up of gap junction(s) at different sites throughout the body in terms of connexin sub-unit composition.

5 Some connexin proteins are however more ubiquitous than others in terms of distribution in tissue. One of the most widespread is connexin 43. ODN's targeted to connexin 43 are therefore particularly suitable for use in the present invention.

10 It is also contemplated that ODN's targeted at separate connexin protein be used in combination. For example, ODN's targeted to connexin 43, and one or more other members of the connexin family can be used in combination.

The ODNs for use in the invention will generally be unmodified phosphodiester oligomers. They will also usually be no longer than 36 oligonucleotides in length with a 30 mer ODN being particularly suitable.

15 The precise sequence of the ODN used in the invention will depend upon the target connexin protein. For connexin 43, the applicant's have found ODN's having the following sequence to be particularly suitable:

20 GTA aTT gCC gCA GGA GGA ATT GTT GCT GTC; and  
GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT

ODN's directed to other connexin proteins can be selected in terms of their nucleotide sequence by any convenient, and conventional, approach.

25 For use in the invention, the ODN(s) require site-specific delivery. They also require delivery over an extended period of time. While clearly the delivery period will be dependent upon both the site at which the downregulation is to be induced and the therapeutic effect which is desired, continuous delivery for 24 hours or longer will  
30 often be required.

In accordance with the present invention, this is achieved by inclusion of the ODN(s) in a treatment formulation which comprises the ODN(s) and a sustained release vehicle. While any such vehicle which is non-toxic may be employed (depending  
35 upon the desired ODN release profile), it is preferred that a gel or gel-based vehicle be used.

Pluronic-type gels have been found to be particularly suitable. The presently most preferred vehicle is the gel Pluronic F-127 (BASFV Corp). This gel is preferred as it is liquid at low temperatures but rapidly sets at physiological temperatures, which  
5 confines the release of the ODN component it contains to the site of application or immediately adjacent that site.

In addition to the ODN and sustained release vehicle, the formulation may also contain a surfactant. The purpose of including a surfactant is to assist the ODN  
10 component in cell penetration.

Where a surfactant component is included, any conventional non-toxic surfactant can be selected. An example is DMSO.

15 Once prepared, the formulations of the invention have utility in any therapeutic/cosmetic approach where a transient and site-specific interruption in cell-cell communication is desirable. These include in treating neuronal damage (where the damage is to be localised as much as possible), in the promotion of wound healing and in reducing scar formation following, for example, cosmetic  
20 surgery.

Various aspects of the invention will now be described with reference to the following experimental section which will be understood to be provided by way of illustration only and not to constitute a limitation on the scope of the invention.

25

## **EXPERIMENTAL**

### **Materials and Methods:**

#### **30 Antisense application:**

30% Pluronic F-127 gel (BASF Corp) in phosphate buffered saline (molecular grade water) was used to deliver unmodified  $\alpha 1$  connexin (connexin 43) specific anti-sense ODNs to the developing chick embryo (Simons, *et al.*, 1992). Chick embryos were  
35 incubated at 38°C and staged according to Hamilton and Hamburger stages. Eggs were windowed and the vitelline and amniotic membranes over the area to be

treated were opened using fine forceps. After anti-sense application eggs were sealed with tape and replaced in the incubator for 48 hours at which time most experiments were analysed, the exception being for the time course analysis of  $\alpha 1$  connexin "knockdown" and recovery.

5

Pluronic gel is liquid at low temperatures, 0-4°C, but sets when dropped onto the embryo at physiological temperature, remaining in place for at least 12 hours. The gel has the additional advantage of being a mild surfactant and this, used either alone or in conjunction with DMSO, appeared to markedly expedite ODN penetration into cells (Wagner, 1994). Addition of an FITC tag to DB1 ODN, viewed using confocal laser scanning microscopy, demonstrated intracellular penetration of the probes. Sequences of deoxyoligonucleotides used are shown in table 1.

15 **Table 1: The Effect on Limb Development of ODN Application Between Stages 8 & 14 of Chick Embryo Development**

Antisense deoxyoligonucleotides to Connexin43

DB1 GTA aTT gCG gCA GGA GGA ATT GTT tCT GTC

CG1 GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT

Control deoxyoligonucleotides

DB1(sense) GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC

DB1(chick) GTA GTT ACG ACA GGA GGA ATT GTT CTC GTC

CV3(random) TCG AAC TGT CAA GAC TGC TAT GGC GAT CAT

Gel only

20 All ODNs were applied at 0.5-1.0 mM final concentration following dose dependent analysis during preliminary experiments covering a range of concentrations from 0.05 mM to 50 mM. General toxicity effects only became apparent with ODN concentrations greater than 10 mM. ODN gel mixtures were prepared from concentrated stock solutions stored at -80°C.

## 25 **Anti-sense sequences**

DB1 is a mouse anti-sense sequence, complementary to bases 1094 - 1123 of the  $\alpha 1$  connexin gene. It has four mismatches with chick  $\alpha 1$  connexin sequence. CG1 is complementary to chick  $\alpha 1$  connexin bases 720-749. Efficacy of this probe was



improved with 1% Dimethylsulphoxide (DMSO) added to the gel. DMSO had not added effect on other anti-sense ODN or control results.

### Control sequences

5 DB1(Chick) is the chick  $\alpha 1$  connexin equivalent of DB1 matching chick  $\alpha 1$  connexin bases 954-983. Analysis however, indicates a high probability of forming stem loop structures ( $G = -7.0$  kcal/mol, Loop  $T_m = 92^\circ$ ) and homodimerisation ( $T_m = 1.5^\circ$ ) and therefore acts as a control sequence. It has been reported that some sense oligonucleotides can form stable DNA triplets (Neckers *et al.*, 1993) inhibiting  
10 transcription. However, this was not apparent with DB1 (sense). Random control sequence with no stable secondary structure ( $G = 1.4$  kcal/mol) and unstable homodimerisation were also used CV3. An additional control applying equal concentration mixture of DB1 and DB1 (sense) gave background levels of defects.

### 15 Monitoring of protein knockdown

Immunohistochemical localisation of  $\alpha 1$  connexin gap junction protein at cell-cell interfaces provides a direct measurement of the anti-sense effect. Anti-peptide  $\alpha 1$  connexin specific antibody probes were used to stain wholemount embryos and the connexin distribution was analysed using confocal laser scanning microscopy  
20 according to established procedures (Green *et al.*, 1995). Control labelling for two other connexins expressed in the developing chick embryo (connexins  $\beta 1$  &  $\beta 2$ ) was similarly carried out, also using sequence specific antibodies (Becker *et al.*, 1995).

## RESULTS

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### Reduction of $\alpha 1$ connexin expression

Using Pluronic F-127 gel to deliver unmodified  $\alpha 1$  connexin specific anti-sense ODNs to the developing chick embryo, protein expression can be interfered with at chosen time points and allows the anti-sense treatment to be targeted to specific  
30 regions of a chick embryo. A droplet of gel containing the anti-sense at a relatively low concentration was placed precisely onto individual embryos. The gel sets and remains in place for at least 12 hours and thus a sustained low dose of anti-sense is maintained in this region. The anti-sense applications were targeted and timed to block junction formation prior to the periods of elevated expression in the limb,  
35 neural tube and face. These times were chosen to optimise the effects of the anti-sense by reducing the expression of new protein rather than being dependent upon

the turnover of protein already in the membranes of the cells of the target tissue. Both DB1 and CG1 ODNs reduced expression of  $\alpha 1$  connexin protein within two hours in the neural tube and limb bud, dramatic within 4-8 hours and persisted at 18-24 hours and 48 hours in some tissues (data not shown). No down regulation of  $\alpha 1$  connexin protein was evident in any of the controls used. Equally, two other members of the connexin family expressed in the chick embryo,  $\beta 1$  connexin and  $\beta 2$  connexin, were unaffected by the  $\alpha 1$  connexin specific anti-sense ODN.

Several parallel controls were run with all of the experiments. These included; DB1 sense, DB1 anti-sense and DB1 sense combined, DB1 chick (which forms stem loop structures with itself), random ODNs CV3, Pluronic gel alone, Pluronic gel with DMSO and PBS alone). None of the controls had a noticeable effect on  $\alpha 1$  connexin protein expression.

#### UTILITY

Thus, in accordance with the invention, there are provided formulations by which cell-cell communication can be downregulated in a transient and site-specific manner. The formulations therefore have application in methods of therapy and in cosmetic treatments.

The delivery of the ODN component of the formulation for an extended period (24 hours or longer) is a particular advantage in treating neuronal damage. This is because, in most instances of direct physical neuronal insult, neuronal cell loss extends well beyond the site of actual injury to the surrounding cells. This secondary neuronal cell loss occurs within 24 hours of the original injury and is mediated by junction gap cell-cell communication. Downregulation of connexin protein expression therefore blocks or at least downregulates communication between the cells and minimises secondary neuronal cell damage.

Equally, in instances of other tissue damage (particularly wounds) the formulations of the invention have been found effective in both promoting the wound healing process and in minimising scar formation. The formulations therefore have clear benefit in the treatment of wounds, whether the result of external trauma or surgical intervention.

It will further be appreciated that the above description is provided by way of example only and that modifications can be made, both in terms of the specific ODN's and sustained release vehicles employed without departing from the scope of the present invention.

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